The cell wall of *Paracoccidioides brasiliensis*: insights from its transcriptome

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ABSTRACT. The cell wall of a human pathogenic fungus is in contact with the host, serves as a barrier against host defense mechanisms and harbors most fungal antigens. In addition, cell wall biosynthesis pathways have been recognized as essential to viability and as specific drug targets. *Paracoccidioides brasiliensis* is a dimorphic fungus that presents mycelium morphology in the free environment and causes infection in a yeast form. The morphogenetic conversion is correlated with changes in the cell wall composition, organization and structure. Based on transcriptome analysis, the enzymes involved in the biosynthesis and remodeling of cell wall polysaccharides, as well as several cell wall-associated molecules of *P. brasiliensis*, were identified and addressed in further detail.

Key words: *Paracoccidioides brasiliensis*, Cell wall, Dimorphism, Cell wall-associated enzymes

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INTRODUCTION

The cell wall of a fungal pathogen is in permanent contact with host cells; it acts as a filter and a reservoir for molecules, such as antigens and enzymes, thus also having an active role during infection (Latgé, 1999). The cell wall is apparently a dynamic structure, whose constituent polymers are continuously modified and rearranged during its biosynthesis (Popolo et al., 2001). *Paracoccidioides brasiliensis* is a dimorphic fungus that causes paracoccidioidomycosis. As with other pathogenic fungi, culture conditions influence the cell wall composition and morphology of *P. brasiliensis* (Kanetsuna et al., 1969; San-Blas and Vernet, 1977; Da Silva et al., 1994). We examined cell wall metabolism in *P. brasiliensis*, by searching for genes in its transcriptome that could be involved in the construction and maintenance of cell wall polymers. We also looked for cell wall-associated molecules in this fungus and how they were differentially expressed in mycelial and yeast forms.

STRUCTURE AND CELL WALL COMPOSITION OF *PARACOCCIDIOIDES BRASILIENSIS*

In *P. brasiliensis*, as in other fungi, lipids, chitin, glucans, and proteins are the main constituents of the cell wall in both mycelial and yeast forms. The lipid (5 to 10%) and glucan (36 to 47%) content of the cell wall is similar in the two forms. The yeast form has a larger amount of chitin (37 to 48%) than the mycelial form (7 to 18%). The mycelium has a higher concentration of proteins (24 to 41%) when compared to the yeast cells (7 to 14%) (Kanetsuna et al., 1969). The main polysaccharide of the yeast cell wall is α-glucan, whereas the polysaccharides of the mycelium wall are β-glucan and galactomannan (this latter corresponding to about 6% of the total). The yeast α-glucan also contains small amounts of α-1,3- or α-1,6-glycosidic linkages. On the other hand, the mycelial cell wall β-glucan contains mainly β-1,3-glicosidic linkages, with small amounts of the β-1,6-glicosidic linkages. The degree of polymerization of β-glucan is 30 glucose residues joined through β-1,3-bonds and side branches with β-1,6-linkages (Kanetsuna et al., 1972). Electron microscopy studies have shown that the mycelium has a single-layered cell wall with chitin and β-glucan fibrils, whereas the yeast has three layers (Carbonell and Rodriguez, 1968). The inner surface is chitin with some β-glucan and the outer surface is formed by α-glucan (Carbonell, 1972). Low amounts of galactose and mannose were observed in the cell wall of the mycelium (Kanetsuna et al., 1969), which contained 12 times more disulfide linkages than its yeast counterpart.

Although chitosan has not been identified in the cell wall of the yeast or the mycelium phase, the gene encoding chitin deacetylase (*cda*), the enzyme that converts chitin to chitosan, was found to be over expressed in yeast, which was confirmed by cDNA microarray data (Felipe et al., 2003, 2005). In addition, the *fluG* gene, which initiates conidiophore development in *Aspergillus* (Lee and Adams, 1994), was found in the mycelium phase, indicating the existence of conidia (Table 1).

The structure of alkali-extracted water-soluble cell wall polysaccharides (F1SS) from both phases of *P. brasiliensis* has been studied. The F1SS polysaccharide from mycelium consists of a trisaccharide repeating unit of →6)-[α-Gal*f*-(1→6)-α-Man*p*-(1→ 2)]-α-Man*p*-(1→. The F1SS polysaccharides of the yeast phase maintains 10% of the structure of the mycelium phase, but the main structure contains a disaccharide repeating unit of →6)-[α-Man*p*-

Table 1. Enzymes associated with biosynthesis and remodeling of the cell wall of *Paracoccidioides brasiliensis*.

PbAEST	Ortholog name	Product	References	Remarks
Synthases				
5198	*Pbfks1*	1,3-β-glucan synthase	Pereira et al. (2000)	Synthesize glucan polymer
4988	*Pbags*	1,3-α-glucan synthase		Expressed in yeast phase
	rho1, rho2	RHO	AY392528, AY496954	Regulate 1,3-β-glucan synthase
0265, 944	*rho3, rho4*	RHO		Regulate 1,3-β-glucan synthase
1147, 1927, 3456, 5473, 954	*Pbchs1, Pbchs2, Pbchs3, Pbchs4, Pbchs5,*	Chitin synthase	Niño-Vega et al. (1998, 2000)	Expression changed during dimorphic transition
3958	*chs6*	Chitin synthase		Expressed in the mycelium phase
1011, 742, 4968	*pgi, gfa* and *gna*	*Phosphoglucose isomerase, ketol-isomerase and glucosamine-6-phosphate acetyltransferase*		Precursors of the chitin
Remodeling enzyme and others				
381	*dpm1*	Dolichol phosphate mannose synthase		Synthesize Dol-P-Man from GDP-mannose (Ernst and Prill, 2001)
	Pbymnt	Mannosyltransferase	Costa et al. (2002)	Expressed preferentially in the yeast phase
2980	*pmt1*	Mannosyltransferase		Required to dimorphism (Ernst and Prill, 2001). Present in yeast phase
3607	*ktr1*	Mannosyltransferase		
1063	*ktr3*	Mannosyltransferase		Present in the mycelium phase
191	*mnn2*	Mannosyltransferase		
3220	*mnn9*	Mannosyltransferase		Present in the yeast phase
202	*bgl2*	Glucosyltransferase		Transglucosylase
1370	*gas1*	Endotransglycosylase		May be involved in extending and rearranging 1,3-β-glucan chains (Popolo and Vai, 1999)
5441	*crh1*			Localized at the cell surface, near chitin-

Continued on next page

Table 1. Continued.

PbAEST	Ortholog name	Product	References	Remarks
				rich areas (Rodríguez-Peña et al., 2000)
1527, 2375, 1370	*gel1*, *gel2*, *gel3*	β-1,3-glucanosyltransferase	AY380566, AY340235, AY324033	Transglucosylase
	Pbnag1	N-acetyl-β-D-glucosaminidase	Santos et al. (2004)	
1058	*egl*	β-1,3-endoglucanase		
1195	*cts1*	Chitinase		Daughter cell specific expression
1281, 1511	*scw1*, *dse4*	Glucanase		Daughter cell specific expression
0737	*cda*	Chitin deacetylase		Expressed preferentially in the yeast phase
4082	*fluG*			Present in the mycelium phase
	Pbhyd, *Pbhyd2*	Hydrophobins	Albuquerque et al. (2004)	Expression changed during dimorphic transition in *P. brasiliensis*
5473		*Bud neck*		
2516, 567, 2630	*cdc12*, *cdc15*, *imp2*			Ring assembles
528	*plo1*	polo kinase		Ring positioning
951, 1592	*cdc4*, *act1*			Essential ring components
2647, 4574	*cdc11*, *cdc14*			Septation initiation network - SIN components

PbAEST = *Paracoccidioides brasilienses* assembled expressed sequence tags.

(1→2)]-α-Man*p*-(1→ alternated with a trisaccharide repeating block of →6)-[β-Gal*f*-(1→6)-α-Man*p*-(1→ 2)]-α-Man*p*-(1→ (Ahrazem et al., 2003).

CELL WALL OF *PARACOCCIDIOIDES BRASILIENSIS*: DIMORPHISM X VIRULENCE

The cell wall has an essential role in the pathobiology of *P. brasiliensis*. The morphogenetic changes are directly associated with the life cycle of this fungus. It undergoes some molecular rearrangement during the morphogenetic switch from hyphae to the yeast phase (Da Silva et al., 1994). Dimorphism has been cited as a crucial factor in the establishment of infection, as strains unable to differentiate into yeast do not cause disease (Borba and Schäffer, 2002). In addition, *P. brasiliensis* cells presented thicker cell walls after passage in animals than cells subcultured *in vitro* for many years (San-Blas, 1982), suggesting alterations in the cell wall metabolism.

Cell wall polysaccharides, α-1,3-glucan and β-1,3-glucan have been proposed as possible contributors to the dimorphic transition of *P. brasiliensis* (San Blas and San Blas, 1994). A lower α-1,3-glucan content in the cell wall of the yeast form has been correlated with lower virulence (Hallak et al., 1982). *In vitro* culture of virulent *P. brasiliensis* isolates for long periods results in thinner cell walls, loss of virulence and lower α-1,3-glucan levels (San-Blas and San-Blas, 1977). The other main polysaccharide, β-1,3-glucan, has been implicated as an important immunomodulator (Restrepo-Moreno, 1993; Silva et al., 1997). When present together, α- and β-1,3-glucan have been appointed as virulence factors.

A hypothesis formulated by Kanetsuna et al. (1972) and modified by San-Blas and San-Blas (1985) explains the differentiation from mycelium to yeast and vice-versa. By the combined activity of β-glucanase and disulfide reductase, the yeast cell wall is loosened around discrete islets of β-glucan, forming a bud. At 37°C the high activity of disulfide reductase, and higher synthesis of chitin and α-glucan than β-glucan result in the yeast form. At 22°C the disulfide reductase has low activity, α-glucan synthesis occurs at low rates and long β-glucan fibrils are formed at the budding sites.

IDENTIFICATION OF CELL WALL-ASSOCIATED MOLECULES

Classes of cell surface proteins have been described. Proteins released by extraction from intact cells with reducing agents are named cell wall proteins, whereas proteins linked to β-1,3-glucan through a connecting β-1,6-glucan moiety are named GPI-dependent cell wall proteins. The *P. brasiliensis* transcriptome presents expressed sequencing tags (ESTs) encoding for both kinds (Table 1).

Electrostatic forces are involved in the attachment of microorganisms to several types of surfaces (van Oss et al., 1986), and they may be relevant to the interaction between the microorganism and the host cell (Hesketh et al., 1987). Sialic acid residues are constituents of many glycoconjugates and are the major ionogenic compounds that contribute to the negative surface charge of many cell types (Schauer, 1982). Yeast and mycelial forms of *P. brasiliensis* express surface sialic acid units (Soares et al., 1993). Analysis of the surface anionogenic groups and sialoglycoconjugate structures of yeast forms suggests that sialic acid residues are the main anionogenic groups on the *P. brasiliensis* surface (Soares et al., 1998).

Hydrophobins are small proteins secreted as monomers that self-assemble into an amphipathic film at hydrophilic/hydrophobic interfaces covering fungal structures (Wösten and de Vocht, 2000). Two hydrophobin single-copy genes (Table 1) are present in the *P. brasiliensis* genome, and Northern blot analysis revealed that both mRNAs are mycelium-specific and highly accumulated during the first 24 h of the mycelium-to-yeast transition (Goldman et al., 2003; Albuquerque et al., 2004).

CELL WALL-ASSOCIATED ENZYMES

Synthases

1,3-β-glucan synthase

In *P. brasiliensis*, 1,3-β-glucan synthase requires uridine diphosphate glucose (UDPG) as the preferred nucleotide precursor to the *in vitro* synthesis of β-glucan (San-Blas, 1979). This reaction is inhibited by GTP and other nucleotides (San-Blas and San-Blas, 1986). To date only one homologue, *Pbfks1* (Table 1), has been cloned and characterized (Pereira et al., 2000). *Pbfks1* has an open reading frame of 5942 bp, interrupted by two putative introns, codifying a predicted protein of 1926 amino acids (212 kDa). The presence of putative regulatory signals suggests a flexible and complex control mechanism for the expression of *Pbfks1*, as described for the homologous *fks1* and *fks2* of *S. cerevisiae* (Mazur et al., 1995). Although the UDPG-binding motif of the 1,3-β-glucan synthase has not been found yet, analogous domains to the UDPG-binding motif of cellulose synthase, delimited by Kelly et al. (1996), were found in *PbFKS1*. Figure 1 presents the alignment of putative domains found in *A. nidulans*, *Acetobacter xylinum* and *P. brasiliensis*.

```
Domain 1

FKSAp   : 861 PMPEPLPVDNMPTFTVLIPHYSEKI 885
              P+P P  VD+ PT  + IP Y E++
BCSAp   : 138 PLPLPDNVDDWPTVDIFIPTYDEQL 162
              P+P P  VD+ PT  + IP Y E++
PbFKS1p: 864 PIPEPVPVDNMPTFTVLIPHYSEKI 888

Domain 2

FKSAp   : 968 LRTRIWSSLRSQTLYRTVSGMMNYSRAIKLLYRVENP 1004
              LR  + S L ++ +   +   + ++R +  ++RV+NP
BCSAp   : 350 LRIPVASGLATERLTTHIGQRMRWARGMIQIFRVDNP  386
              LR  + + L ++ L   I   M ++R +  ++RV+NP
PbFKS1p: 970 LRTRIWASLRSQTLYRTISGFMNYSRAIKLLYRVENP 1006
```

Figure 1. Domain 1 and 2 analogs to the UDPG-binding motif of cellulose synthase. The regions present in cellulose synthase of *Acetobacter xylinum* (BCSAp) (GenBank accession No. SP19449) are identified in 1,3-β-glucan synthase of *Aspergillus nidulans* (FKSAp) (GenBank accession No. U51272) and of *Paracoccidioides brasiliensis* (PbFKS1p) (GenBank accession No. AF148715). + Indicates similar amino acids.

In *P. brasiliensis*, PbFks1p seems to assemble the phosphorylated glucan polymer and extrude it out of the membrane simultaneously, since the PTS-HPr (phosphotransferase system-phosphoryl carrier protein) phosphorylation site motif was found in the predicted protein PbFks1p. Hydropathy analysis putatively classified PbFks1p as an integral membrane protein displaying a catalytic cytoplasmic domain between two transmembrane regions (Pereira et al., 2000). Analysis of particulate preparations of the *P. brasiliensis* suggested that 1,3-β-glucan synthase localizes mainly to the cytoplasmic membrane (Sorais-Landaez and San-Blas, 1993).

1,3-β-glucan synthase is regulated by the RHO GTPases, which are multifunctional regulators that interact with numerous proteins (Douglas, 2001). The role of RHO1p in regulating 1,3-β-glucan synthase has been studied in pathogenic fungi such as *Candida albicans* (Kondoh et al., 1997), *Aspergillus fumigatus* (Beauvais et al., 2001) and *Cryptococcus neoformans* (Tanaka et al., 1999). Two *rho* ESTs, *rho3* and *rho4*, were identified in the transcriptome of *P. brasiliensis*. Although *rho1* (AY392528) and *rho2* (AY496954) have already been cloned, their sequences are still partial. In fact, *rho1*, *rho2*, *rho3*, and *rho4* present the ATP/GTP-binding site motif ([AG]-x(4)-G-K-[ST]) and residue alignment by the CLUSTAL X program estimated similarity of 26 to 42%, indicating that they are distinct genes (Figure 2).

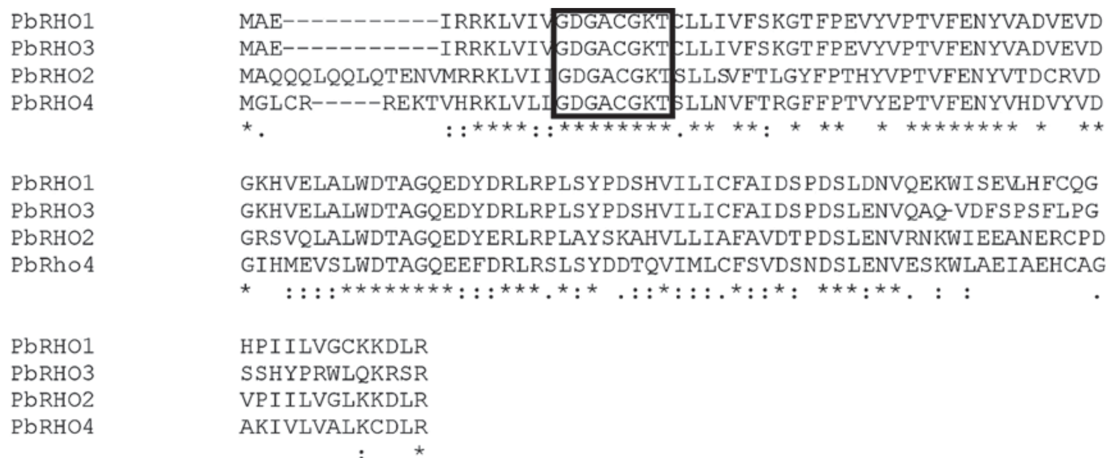

Figure 2. Alignment of the rho genes of *Paracoccidioides brasiliensis*. Asterisks indicate conserved amino acid residues. The symbols (: and .) denote a decreasing order of matching similarity between each corresponding amino acid pair. Amino acids inside the box indicate the ATP/GTP-binding site motif.

RHO1p activity is regulated by the activator ROM1p (Ozaki et al., 1996) and by repressor SAC7p (Schmidt et al., 1997). SLA1p, a protein that is important for actin nucleation, is required for the localization of RHO1p (Ayscough et al., 1999). In addition, RHO1p signals to the actin cytoskeleton through Bni1p, the key component of the polarisome, which binds to the barbed ends of actin filaments and nucleates microfilament assembly (Delley and Hall, 1999; Pruyne et al., 2002). All the genes described above were present in the *P. brasiliensis* transcriptome and are listed in Table 1.

1,3-α-glucan synthase

1,3-α-glucan synthase is the main cell wall neutral polysaccharide of the outer capsule

of the pathogenic yeast phase of *P. brasiliensis* and has been proposed as a virulence factor in this fungus (San-Blas et al., 1977), as well as in *Blastomyces dermatitidis* (Hogan and Klein, 1994) and *Histoplasma capsulatum* (Klimpel and Goldman, 1988). We have identified the 1,3-α-glucan synthase gene, Ags, in the *P. brasiliensis* yeast transcriptome (Table 1). To our knowledge, few 1,3-α-glucan synthase genes have been isolated. Figure 3 shows the alignment of 1,3-α-glucan synthases that presented homology on BLAST analysis (Altschul et al., 1990). Although only a short region has been sequenced, 15% identity and 46% similarity were observed.

```
Af.AGS1      WAGFLFVLSHLSKKHSWILPVFACGLGAPRWAQIWWGVSGIGYYMPWVAGGLTGGALASR
Af.AGS2      WAALLYLFGYLSKSHSWIIPVFACGLGAPRWAQVLWGVSGIGLYVPWAGGDLAG-ALVSR
Pb.AGS       WVVLLGFLSYLSKDHSWIMPLFAIGLGAPRWAQIWWGTSGIGQHVPWAHGYVAG-ALVSR
Nc.AGS       WCLLLWLFAYFTKTHPWIVPLFAIGLGAPRWAQMLWATSGIGLYLPWCGTVVLS-AIISR
Sp.AGS1      WALLLGVLAWISRTHSWIICVFGVGLGAPRWLQQFWATSNIGLYLPWAG--YSG-PYLGR
Sp.MOK1      WALLLGVLAWISRTHSWIICVFGVGLGAPRWLQQFWATSNIGLYLPWAG--YSG-PYLGR
Sp.MOK13     WIIMLMFLGRKSLTHSWLLPVFGVGLGSPRWIQMMWGTSNIGVYLPWAG--VAG-PIVGR
Sp.MOK14     WLIIFYVLRKLSNKHTWMVPVLGLGFGAIKWMHVFWGTSNVGIFLPWAG--IAG-PYLSR
Sp.MOK12     WAVLLSVIKILSLNNVWFPVIFGLGLICPRWCLEFWSSSGLGINLPWAG--KAS-ALLTK
             *  :: .:   :  : *:  ::. *: . :*    *. *.:*  :**      . .   :

Af.AGS1      SIWLWLGVLDAIQGLGFGMILLQTLTRMHMCFTLIVCQVLGSIATICARAFAPNNVGPGP
Af.AGS2      SLWLWLGVLDSLQGLGFGMILLQTLTRMHICFTLLASQVLGSIATICARAFAPNNIGPGP
Pb.AGS       SLWLWLGVLDAMQGVGFGMILLQTLTRFHICFTLLAAQALGSIATICARTFSPNKIGPGD
Nc.AGS       CLWIWLGLLDTVQGVGLGMVLLLTLTRQHVAATLIGAQFLGAVFMMLARATAPDKDGPGD
Sp.AGS1      TLWLWLGVLDAIQSVGIGMILLQTLTRRHVASTLMTGQIVGAVATMIGRGASPNREGPAN
Sp.MOK1      TLWLWLGVLDAIQSVGIGMILLQTLTRRHVASTLMTGQIVGAVATMIGRGASPNREGPAN
Sp.MOK13     ILWIWLGVLDSVQGVGVGMILLQTLTRRHIATTLIAGQIIGTLTSMLARATAPNRLGPGL
Sp.MOK14     ALWLWLGILDSIQGIGNGLILLQTLSRRHVTNTLMISQLAGSATSILARFVSPTKTGPAN
Sp.MOK12     SVWLLLALWDGIQGVGVGVMLLQTLARDHVAFTLMLAQVISCITIMIAKPSLPVS---DR
              :*: *.: * :*.:* *::** **:* *:  **:  *  .    : .:   *

Af.AGS1      ISPDPT----YGAS-AVANAWFWIALIFQLLICAGFLLFFRREQLAKP
Af.AGS2      ISPDLT----AGVS-SVANAWFWIAIFFQLLICAGFLRFFRKEQLSKP
Pb.AGS       IHPDIS----GGIS-AIWTAWFWICLFFQLAICAGFYTFFRKEQLSKP
Nc.AGS       VFPDFS----AGVMPGLGRPWFWVVLGLQLVLPIGFFKFFRKEQVAKP
Sp.AGS1      VFIDFTKWNHGDGSSILASAPFWINIICQLAICVGYLAFFRRENLSRP
Sp.MOK1      VFIDFTKWNHGDGSSILASAPFWINIICQLAICVGYLAFFRRENLSRP
Sp.MOK13     VFLDLTSWRFEDGAKIFRSAPFWICLISQIAVSAGYLLFFRRENLSRP
Sp.MOK14     VFPDLTGYTPVDRAKPVANAPFWICLILNVALCIMYLRCYHRENISRP
Sp.MOK12     VFPNLGAWNPSEGPGPCASPCFYIALICQFVAVGGLLYHYRKSQLA--
             :  :               . *:: :  :.         :::.:::
```

Figure 3. Alignment of the 1,3-α-glucan synthases. The sequences used were: from *Aspergillus fumigatus*, Af.AGS1 (GenBank accession No. AAL28129.1) and Af.AGS2 (GenBank accession No. AAL18964.1); from *Paracoccidioides brasiliensis*, Pb.AGS; from *Neurospora crassa*, Nc.AGS (GenBank accession No. CAE76407.1), and from *Schizosaccharomyces pombe*, Sp.AGS1 (GenBank accession No. CAA22822.1), Sp.MOK1 (GenBank accession No. BAA34054.1), Sp.MOK12 (GenBank accession No. CAC37503.1), Sp.MOK13 (GenBank accession No. BAA76559.1), and Sp.MOK14 (GenBank accession No. BAA76560.1). Asterisks indicate conserved amino acid residues. The symbols (: and .) denote a decreasing order of matching similarity between each corresponding amino acid pair.

Chitin synthase

Membrane-bound chitin synthase catalyzes the polymerization of GlcNAc (N-acetyl-

β-D-glucosaminidase) from cytosolic UDP-GlcNAc into polysaccharide chains that are extruded to the cell wall (Ruiz-Herrera, 1992; Gooday, 1995). Chitin synthesis in fungi is a complex process (Horiuchi and Takagi, 1999), regulated by multigene families and involved in distinct physiological processes (Cabib, 1991; Gaughran et al., 1994). Although it has been possible to express *chs* genes in a heterologous host, these transmembrane proteins have not yet been produced as soluble recombinant proteins (Bulawa et al., 1986; Silverman et al., 1988).

In *P. brasiliensis*, five chitin synthases have been identified by PCR amplification of conserved *chs* gene domains (Niño-Vega et al., 2000), two of which have been isolated, namely *Pbrchs2* (GenBank accession No. Y09231) and *Pbrchs4* (GenBank accession No. AF107624). The deduced amino acid sequence of CHS2p consists of 1043 residues and is highly homologous to other class II fungal chitin synthases. It presents a highly variable region at the cytosolic amino-terminal region, which may be related to a possible zymogenic nature, and a putative catalytic region close to seven membrane-spanning regions at the carboxyl terminus (Niño-Vega et al., 1998). The gene *Pbrchs4* codes for a predicted protein, consisting of 1744 amino acids, with a C-terminal domain homologous to chitin synthases and an N-terminal domain with homology to myosin motor-like domains, although the latter does not present classical signatures (Niño-Vega et al., 2004).

Despite the fact that yeast cells contain more chitin than do hyphae, the levels of mRNA for *Pbrchs1* (GenBank accession No. AF107622), *Pbrchs2*, *Pbrchs3* (GenBank accession No. AF107623), *Pbrchs4* and *Pbrchs5* (GenBank accession No. AF107625) were higher in the former (Niño-Vega et al., 2000), suggesting that post-transcriptional regulation of *chs* gene expression is important for morphogenesis.

We identified a new chitin synthase (*Pbrchs6*), which is present only in the mycelium phase of *P. brasiliensis*. An update of the phylogenetic tree (Niño-Vega et al., 2004) with all CHS predicted proteins of *P. brasiliensis* is presented in Figure 4. It was constructed by multiple sequence alignments with the Clustal X program, following the neighbor-joining method (Thompson et al., 1997). Robustness of branches was estimated using 100 boot-strapped replicates. The amino acid sequences were viewed with the TreeView software. The tree shows that *Pbr*CHS6 probably belongs to class VI, since it is branched together with *Af*CHSD, a chitin synthase of this class (GenBank accession No. U62614) from *A. fumigatus* (100% bootstrap confidence levels of branches). *Pbr*CHS2 and *Pbr*CHS4 belong to class II and VII, respectively. The *chs* genes from other fungi were grouped in their respective classes.

Remodeling enzymes

Manosyltransferase

Some cell wall proteins are glycosylated on serine or threonine amino acids by the addition of mannose residues. Dolichol phosphate mannose synthase (DPM1p) synthesizes Dol-P-Man from GDP-mannose, which is the substrate for protein mannosyltransferases (PMT) (Ernst and Prill, 2001). O-glycosylation in many fungal species is initiated in the endoplasmic reticulum by PMTp, which transfer the first mannose to serine or threonine residues, and is completed by mannosyltransferases (MNTp) in the Golgi compartment by the concerted action of a range of mannosyltransferases, including MNT1p, KTR1p, KTR3p, and MNN1p, which attach further mannose residues to the first O-linked mannose sugar (Gow et al., 1999; Ernst

Figure 4. Phylogenetic tree of fungal chitin synthases. Sequences were aligned using CLUSTAL X programm. Sequences were taken from GenBank. Ca, *Candida albicans*; Wd, *Wangiella dermatitidis*; An, *Aspergillus nidulans*; Ao, *Aspergillus oryzae*; Bg, *Blumeria graminis*; Gg, *Glomerella graminicola*; Um, *Ustilago maydis*; Pb, *Phycomyces blakesleeanus*; Pbr, *Paracoccidioides brasiliensis*; Nc, *Neurospora crassa*; Sc, *Saccharomyces cerevisiae*; Af, *Aspergillus fumigatus*; Mg, *Magnaporthe grisea*.

and Prill, 2001). PMTp1 and MNTp1 are critical for cell wall structure, fungal adhesion and virulence (Gozalbo et al., 2004). In addition, Pmt is required for dimorphism of *C. albicans* (Ernst and Prill, 2001). Although they seem functionally redundant in the elongation of the second and third mannose residues of the O-linked mannan oligosaccharide, *Pby*mnt1, *Pb*ktr1 and *Pb*ktr3, which are present only in mycelium ESTs, were identified in the transcriptome of *P. brasiliensis* (Table 1). The low identity observed among them (Figure 5) could be due to the shortness of the sequences obtained.

The MNN9 protein is involved in the synthesis of *N*-linked outer chain mannan. The *mnn9* knockouts exhibit characteristic phenotypes of defects in cell wall biosynthesis and/or assembly (Wills et al., 2000). The *dpm1*, *pmt1*, *mnn2*, and *mnn9* genes were identified in the

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Pbktr3          ----------------AATATTTCATTTTGAGGGTTGATCGGTTTATTC--TTCCTTTTA  42
Pbktr1          AAAAGGAGGAAGTACACATTCTTCAATGAAATCGCCTACCGACACGTACCATTCGTACCA  100
Pbmnt           AAATACCAATAAAACTGACTCTTTAATTTAAT-ATATATAGATATATAT--CTCCTTCAA  657
                                 *   ** * *   *      *  *     *     ** *   *

Pbktr3          ACACCAGGAGAGAGAGGGAGAGAGAG-AGAGAGAGAGTCGAACAATAAACGAAACAAACA  101
Pbktr1          CTGCCCAACGGGAGAGCAGAAAAGACTAGATCTGAAATGCCACTGCAATCCCAAAGACAA  160
Pbmnt           TTCTTCTGTGCTTGGAAATGTTTGATGCAAAACATGTTTCAAAATTAACTTTTGTCCATA  717
                         *   *         **    *       *   *    **           *

Pbktr3          CCCTTGCCTG-AGTGAACCGGATTTCGATTCCCGG-GCTTCTATATCACTATG-------  152
Pbktr1          CTTTGACTGG-AAGGGACATTCTTGCACATCTCGATACTTCGATGTCAACAAGCTAAAGA  219
Pbmnt           ATTTGGCCGGCAGAAGCCCTTAAAAGAAAATAGGATAATCGGATATTCCACGGTCATGAA  777
                   *  *  * *     *               *    *   ** *      *

Pbktr3          AATTCGGTT----TCTGGGAAGTACCTACGGTACATTCTATTTGTGGTTTTGGG---CCT  205
Pbktr1          AGCCCGAAG----GCTACGAGG-AGGAGCAGGATTGACTATCCGTTGTTCTCTGGTTTCT  274
Pbmnt           AATCCGGGTGTAATCCGCAGAGAAATTAGAGATCAAAATATCAATTCAAATATCA--ATT  835
                *   **        *      * *      *       ***   *     *        *

Pbktr3          TACAATTCTACATTTTAT----ATCCTCCTCCTCGTTACCTCTTCCAAACGCCAACAATG  261
Pbktr1          CGCCAGCGTACTCGTTCTCACTATTGTAGCAATAGTAGCGTATAAAGGTCGTCGATCATG  33 4
Pbmnt           CAGTAGTTTTCACTTTTCAGACATGAGATTCAACGCTATTCCCGTTTGATAGTGAGAGTA  895
                    *   * *   **      **          *                   *   *

PbKT3           TCGTATC-GAACCTCAAGCCAGGAGCGATTA--------AACCTGACTTTAGTTCTCTGT  312
Pbktr1          CTGTCTCAGAACGTCCAGCGTGGACAGTGTGCTCTACATATCGTGACGAAAGTGGTCAAC  394
Pbmnt           TTATTATACGGTGTGCGGTGTTTAT--TTTAGACGAAGGGACCAGAGTTTA-TCATGGGT  952
                   *         *   *     *     *           *  **    * *  *

PbKT3           CTCAAT------CACTATTTTCAGAGAAGTCTAC-TGAAATCCTAACA----------GT  355
Pbktr1          AAAAAAGCAG--CACTGAAAACGAAGAAGACTATATGGGATATAAATACAATGCGGTCAA  452
Pbmnt           TTAAAAGTAAGCCGTCCCATCTCAGAAACCATGTTCAGTTTTCTTCAATCCACGCTCAA 1012
                   **       *        *  ****  *    *    *      *

PbKT3           TTTATTGGCCTCGGCTCTAGC--TCTC-----GCTGCTCCTGCGCAACGTCCCGCCTACG  408
Pbktr1          TGTGTGCATCAAGGGGATAGC--CTTATGTAAGTTATAATATTATATCTTTTTCTCTGCG  510
Pbmnt           TGTAATCTCCATCGCCTTAATAACATCTGCCTACCTTCCTCCCACATTATCATGTCAGCA 1072
                * *      *   *   **      *                   *   *     *  *

PbKT3           AACGAGTAAACGCT------ACCTT----TGTGACCCTAGCCAGAAATGAG-GATGTCTG  457
Pbktr1          GAAATTTTCTTACT------ATTTTCTACTGTAATATTCCCCTTTAGATAG-ACTGCAT-  562
Pb.mnt          AAACCCCGGCCGGCGGAGAAACAAAATGCTCCCACGTCAGCCTTCCATGTTCAAACTCTT 1132
                 *                  *        *   *      **                *

PbKT3           GGATATCTCCAAATCGATCCGTCAGGTGGAGGACCGCTTCAACAGAAATTATCA-CTA-T  515
Pbktr1          --ATATCATTAAACCATTTCACCACATCTACCTCTTCAGAACCTGAAATCGATATCTA-T  619
Pb.mnt          CACTCTCATTTCACTCATTCTCGTCGGCACGATCTCCATCTTCTCATATAAGAACCGAAC 1192
                   * **     *    * *             *  *     *  * **    * * *

PbKT3           GATTGG-GTCTTTATCAACGATAAACCTTTTAACGATGA----ATTCAAGAAGGTTACTT  570
Pbktr1          AGCTCTTACCTCTAAATATCATTATCTACTTGAGTATATCTCTATTCAAAATGAGTGATT  679
Pbmnt           AGCTCTCACCTCACGCAACGTCACATCCTTCAAAAATTTCC--TGTCATCACCATCCACC 1250
                   *     **      *           *  *  **        ***  *

PbKT3           ------CCG--CGCTCGTATCCGGGAAAACCCATTACGGACA------------------  604
Pbktr1          AAAGTACTGTTCGTTCGAAACTCAACAATTACATTTCAGA--------------------  719
Pbmnt           ----TCCTCGAAGCCCATCCCTGAGAAAATCTGGTACAAACTCGGGGCGAAGGGCATTTC 1306
                      *     *  *    *     **      * *  *
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Figure 5. Alignment of the *Pbmnt*, *Pbktr1*, and *Pbktr3* mannosyltransferase genes of *Paracoccidioides brasiliensis*. Asterisks mean identical nucleotides.

transcriptome of *P. brasiliensis* (Table 1). While *mnn2* was present in both phases the others were exclusive to the yeast phase.

Cross-linking of cell wall components

Studies of cell wall chemical organization in *A. fumigatus*, coupled with comparative analysis of *S. cerevisiae* cell wall data, have shown that 1,3-β-glucan branching and chitin-1,3-β-glucan binding are essential exocellular enzymatic steps in cell wall biosynthesis. Cell wall polymers are linked to form an elastic three-dimensional network that acts as a scaffold for the attachment of macromolecules. The final architecture is responsible for the different morphologies of *C. albicans* (Klis et al., 2001) and *P. brasiliensis* (San-Blas et al., 2002).

Enzymes involved in the integration of 1,3-β-glucan have been described. The transglycosidases play an active role in cell wall synthesis and fungal morphogenesis (Beauvais and Latgé, 2001). Mutations in *gel*, *phr* and *epd* all result in alteration of polar hyphal growth. The absence of pH-related genes, PHR1p and PHR2p, changes the composition of the cell wall, produces aberrant morphologies in cells and causes growth defects, thus possibly impairing adaptation to ecological niches with different pH (Navarro-García et al., 2001). We have identified the *bgl2*, *gas1*, *crh1*, *gel1*, *gel2*, and *gel3* transglycosidase genes in the *P. brasiliensis* transcriptome (Table 1).

Hydrolases

Hydrolytic enzymes, such as 1,3-β-glucanases and chitinases, may have roles in the morphogenetic events required for the softening of the cell wall structure (Wessels, 1988). In spite of this, fungal 1,3-β-glucanases have been poorly studied. In contrast to other fungal β-1,3-endoglucanases reported in the literature that are exocellular, the cell wall-associated fungal β-1,3-endoglucanase has been identified in *A. fumigatus* (Mouyna et al., 2002). By comparison, several cell wall-associated and secreted chitinases have been found (Mellor et al., 1994; Hearn et al., 1998). Colman and co-workers have identified 10 genes that show daughter-cell-specific expression (Colman-Lerner et al., 2001). These include the endochitinase *cts1* (Kuranda and Robbins, 1991), and the putative glucanases *scw11* (Cappellaro et al., 1998) and *dse4* (Colman-Lerner et al., 2001). They were also discovered in the *P. brasiliensis* transcriptome (Table 1), and *dse4* was differential for the mycelium phase.

N-acetyl-β-D-glucosaminidase (NAG) is defined as a glycosyl hydrolase enzyme that, in concerted action with chitinase, promotes efficient degradation of chitin by microorganisms (Soto-Gil and Zyskind, 1989; Gooday et al., 1992). Our group has cloned and characterized *Pbnag1*, encoding a NAG of family 20 (Santos et al., 2004).

Mycelial and yeast forms of *P. brasiliensis* were tested for several glycohydrolases through the measurement of their activity to investigate a possible role in the morphogenetic process of the fungus. High levels of β-glucanases, as well as low amounts of α-glucanase, chitinase and maltase were found. Tests for invertase, amylase and lactase were negative. The levels of β-1,3-glucanase were higher in the mycelial form. The shift to the mycelial phase correlated with an increase in the levels of β-1,3-glucanase. The enzyme was present in the cytoplasm, cell wall and culture medium, although enzymatic parameters have been slightly different between extracellular and cell wall-bound enzymes (Flores-Carreón et al., 1979). The

chitinase, 1,3-β-endo- and exoglucanase (*exg1*) genes were identified in the transcriptome of *P. brasiliensis*.

Cell wall precursors

Several new therapeutic targets have been described in the literature, as well as in screening assays to identify respective inhibitors. Phosphoglucose isomerase (*pgi*), glutamine: fructose-6-phosphate amidotransferase (*gfa*) and glucosamine-6-phosphate acetyltransferase (*gna*), which catalyze consecutive reactions in cell wall metabolism, are critical to the synthesis of their precursors. Since alteration in precursor levels results in fungal cell wall abnormalities, leading to cell death, these enzymes are potential targets for antifungal drugs (Selitrennikoff and Nakata, 2003). These, and several other genes involved in aminosugar metabolism, were identified in the transcriptome of *P. brasiliensis* (Table 1).

CONCLUDING REMARKS

Despite our poor knowledge concerning *P. brasiliensis* cell wall metabolism, especially concerning the biochemical and genetic control mechanisms, we found genes encoding cell wall-related synthases, as well as remodeling enzymes, among others. In a comparison with the better studied *S. cerevisiae* cell wall biogenesis, which seems to mobilize about 1200 genes, some of them were also found in the *P. brasiliensis* transcriptome, such as those encoding mannosyltransferases, glucan synthases and chitin synthases. Genes with specific roles in the cell wall of *S. cerevisiae* present homologies in the *P. brasiliensis* transcriptome. This fact suggests similar mechanisms in the construction of the cell walls of *P. brasiliensis* and *S. cerevisiae*. Elucidation of the respective biological processes and functional mechanisms regulating the synthesis, organization and environmental interactions of this complex structure is required for a fuller understanding of this fungus.

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